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AWARD NUMBER: DAMD17-03-1-0471

TITLE: Anti-Cancer Drug Discovery Using Synthetic Lethal Chemogenetic (SLC) Analysis

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REPORT DATE: July 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2006	Annual Summary	1 Jul 2003 – 30 Jun 2006

4. TITLE AND SUBTITLE

Anti-Cancer Drug Discovery Using Synthetic Lethal Chemogenetic (SLC) Analysis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0471

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mount Sinai Hospital
Toronto, Ontario, Canada M56 1X5

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

I am developing a novel cell-based small-molecule screening approach that can identify inhibitors of any non-essential protein function through a surrogate synthetic lethal phenotype in the baker's yeast, Saccharomyces cerevisiae. Synthetic lethality (SL) is a form of genetic enhancement in which two mutations are lethal in combination, but the corresponding individual mutants are viable. Thus, a sensitized yeast strain carrying a mutation that is synthetic lethal with a gene of interest will be inviable in the presence of a chemical inhibitor of the target protein. Systematic genome-wide genetic screens can simultaneously determine all the synthetic lethal genetic interactions for a given gene deletion in yeast. I will adopt this strategy to determine the SL partners for the yeast genes SCH9, the yeast homologue of the human oncogene AKT, and TEP1, the yeast equivalent of the human tumor suppressor PTEN. Selected confirmed synthetic lethal mutants will be used as sensitized strains to screen a commercial small-molecule library for inhibitors of the corresponding proteins. I have developed a yeast-based high-throughput screening platform to screen the Maybridge small-molecule library. Compounds derived from the initial chemical genetic screen will be validated biochemically and, ultimately, tested on mammalian cells for activity against the human homologues.

15. SUBJECT TERMS
high-throughput screening, genome-wide genetic analysis, synthetic lethality, chemical genetics

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	13	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

Project summary:

Cell-based screening relies on an observable phenotype to identify potential hits. Unfortunately, only a small fraction of metazoan genes reveal an easily identifiable phenotype when deleted. For example, only 40% of *Saccharomyces cerevisiae* genes exhibit a growth defect when deleted in haploid cells at standard growth conditions (Winzeler, Shoemaker et al. 1999). Similarly, in the model organism *C. elegans*, approximately half of null mutations exhibit a wild type phenotype (Park and Horvitz 1986). In order to survey the maximum number of chemical-genetic interactions, an easily observable reporter phenotype is required.

I am developing a novel cell-based small-molecule screening approach that can identify inhibitors of any non-essential protein function through a surrogate synthetic lethal phenotype in the baker's yeast, *Saccharomyces cerevisiae*. Synthetic lethality (SL) is a form of genetic enhancement in which two mutations are lethal in combination, but the corresponding individual mutants are viable. Thus, a sensitized yeast strain carrying a mutation that is synthetic lethal with a gene of interest will be inviable in the presence of a chemical inhibitor of the target protein. Systematic genome-wide genetic screens have been developed to simultaneously determine all the synthetic lethal genetic interactions for a given gene deletion in yeast. I will adopt this strategy to determine the SL partners for the yeast genes *SCH9*, the closest yeast homologue to the human oncogene AKT, and *TEP1*, the yeast equivalent of the human tumor suppressor PTEN. Selected synthetic lethal mutants will then be used as sensitized strains to screen commercial small-molecule libraries for inhibitors of the corresponding proteins.

Large-scale library screening requires concurrent development of a yeast-based high-throughput screening platform. Compounds derived from the initial chemical genetic screen will be validated biochemically and, ultimately, tested on mammalian cells for activity against the human homologues. To achieve this, four main aims will be pursued:

Aim 1. High throughput infrastructure development (months1-9).

1. Generate drug-sensitive (DS) parental strains through inactivation of the yeast pleiotropic drug resistance (PDR) network.
2. Synthetic Genetic Array (SGA) analysis of PDR mutants to ensure minimal overlap with the yeast genome.
3. Screening format optimization: liquid vs. solid-phase, growth time, media, etc.
4. High throughput automation development.
5. Wild type screen to establish baseline activity for each compound.

Aim 2. Development of the synthetic lethal chemogenetic screen (months 9-20). I

1. SGA analysis on *sch9Δ* and *tep1Δ* mutants.
2. Confirmation of genetic interactions.
3. SGA analysis of resulting synthetic lethal interactors to exclude shared interactions between sensitized strains other than the target gene.
4. Construct sensitized strains in DS background.
5. Pilot screen on small-scale library to validate method.

6. Full scale Maybridge library screen against sensitized strain.
7. Activity confirmation and construction of "hit compound" mini-array for subsequent testing against sensitized strain pool.

Aim 3. Mechanism of Action (months 18-30).

1. Characterization of phenotype for *SCH9* inhibitors using a Coulter Channelizer.
2. In vitro biochemical assays for inhibition of kinase activity (*SCH9*), or phosphatase activity (*TEP1*).
3. Chemical-genetic fingerprinting: Test candidate compounds in competitive growth assays against the yeast deletion set for compendium profiling.
4. Pairwise screens of candidate compounds for synergistic activity.

Aim 4. Survey compounds against human breast cancer cell lines (months 30-36).

1. Establish human breast cancer cell lines.
2. Growth inhibition assays using compound "hits" from yeast screen.
3. Biochemical assays of gene function.

BODY

Progress

Year One:

Developed DS parental strain
SGA analysis on DS parental strain.
SGA Analysis on *sch9Δ* mutant. Confirmation of 8 novel interactions.
Optimized screening format: 96-well, liquid assay in HEPES buffered SC media.
Developed semi-automated high throughput screening platform at McMaster University.
Screened parental DS strain against Maybridge small molecule library.
Developed analogue-sensitive kinase allele of *SCH9* (*sch9-AS*).
Demonstrated surrogate SL phenotype proof-of-principle using *sch9-AS* sensitized strains.
Screened Maybridge small molecule library against *rpl9bΔ* sensitized strain.

Year Two:

Developed automated high-throughput screening platform at the Samuel Lunenfeld Research Institute.
Developed Chem-Grid database to analyze HT screen data (differential activity scores, hierarchical clustering, visual representation of data).
Cherry –picked a mini-array of putative "hit" compounds from the Maybridge library and tested for differential activity using a dose response assay.
SGA analysis of *tep1*Δ mutant. Confirmed a single genetic interaction with *fun12*Δ.
Screened *SCH9* SL reporter strains against LOPAC and ChemDiv libraries.
Pilot assay for chemical-genetic fingerprinting.

Year Three:

In order to exhaustively define the genetic interactions surrounding *SCH9* and *TEP1*, I undertook a DSLAM analysis of the corresponding mutants against the deletion set. DSLAM is a modified SGA analysis that uses the heterozygous diploid yeast collection and relies on transformation of plasmid DNA and recombination to create the double mutants (Pan, Yuan et al. 2004). The assay is a liquid growth competition that is analyzed by microarray. The advantages of DSLAM are decreased genetic suppression of starting strains due to heterozygosity, fewer false-positive interactions from mating defective mutants and increased signal sensitivity from the microarray.

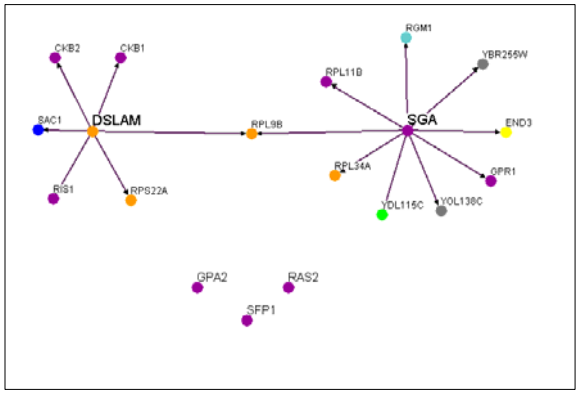

Figure 1. Comparison of *sch9*Δ genetic interactions using SGA and DSLAM. Interacting genes are denoted by circles. Edges connect the assay types as shown. Nodes with multiple edges were shared between screens. Nodes without edges are published genetic interactions that did not appear in either screen.

DSLAM produced 5 new confirmed hits, including an additional ribosomal subunit, *RPS22A* (Fig 1.). Interestingly, there was virtually no overlap between the two assay types. Additionally, DSLAM failed to uncover any of the published genetic interactions. It is clear that these two genetic techniques are powerful, complementary systems for probing genetic interactions on a genome-wide scale, though the coverage is still incomplete.

I next undertook a DSLAM analysis of *tep1*Δ. Previous SGA analysis only uncovered a single genetic interaction between *tep1*Δ and *fun12*Δ. DSLAM produced an additional 13 interactions (Table 1.), but did not recapitulate the interaction with *fun12*, further indicating that the two assays complement each other.

Table 1: Putative genetic interactions with *tep1*Δ.

Known ORF	MRP20	CDH1	KRE21	RSC2	LEU3	MTF1	SMC5	IRA2
Unknown ORF	YNL127W	YNL129W	YNL147W	YOL146W	YOR309C			

The *tep1* DLSAM interactions are being confirmed by mating and tetrad dissection currently.

In order to increase our chemical space and diversity for the synthetic lethal assay, we acquired an aliquot of the 2000 compound Spectrum library from MicroSource. This library is biased toward natural products and provides a further platform for studying chemical synthetic lethal interactions. A preliminary screen of *rpl9b*Δ against the Spectrum library produced a single differential hit compared to the wildtype strain: Clorpropham, an insecticide (Fig 2.).

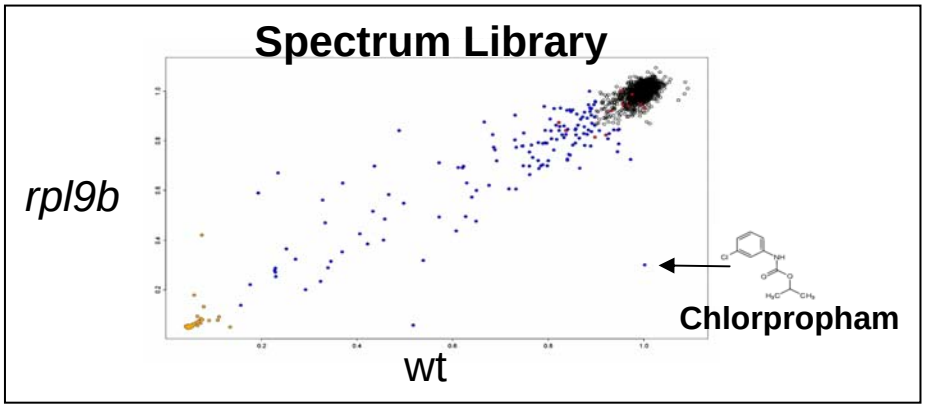

Figure 2. Spectrum Library differential screen against *rpl9b*Δ. Wildtype (wt) or mutant (*rpl9b*Δ) cells were screened against the Spectrum small molecule library at 20μM concentration. Replicates are plotted on the corresponding axes. Activity is reported as normalized residual absorbance compared with DMSO controls. Individual compounds are represented by circles. Bioactive compounds (Z>4) are colored blue and cytotoxic compounds (<2SD from cycloheximide control) are shown in orange. Inactive compounds are open circles and red circles did not replicate within 20%. The molecule with greatest differential activity is denoted by an arrow.

Unfortunately, Clorpropham had no effect on a *gpr1*Δ mutant strain, suggesting that *SCH9* is not the target (data not shown). We have ordered Chlorpropham as part of a restock and can perform a secondary, size phenotype assay to test *SCH9*as a target.

In order to demonstrate the proof of principle for synthetic lethal chemogenetic analysis, I turned to a system in which the target had been previously determined. The immunosuppressant drug Cyclosporin A forms a complex with the cyclophillins and inhibits the phosphatase, calcineurin (Liu, Farmer et al. 1991). The yeast gene *CNB1* encodes the homologue for the regulatory subunit of calcineurin, the functional target of Cyclosporin A. Calcineurin is a non-essential gene in yeast (and humans) and the genetic interactions for *CNB1* are well described (Parsons, Brost et al. 2004), thus Cyclosporin A makes an excellent model system to study surrogate genetic interactions using small molecules. Cyclosporin A is contained within the Spectrum library, thus providing a test case for screening sensitized strains as reporters for *Cnb1* inhibition. A preliminary screen of *fks1*Δ showed a strong differential activity against Cyclosporine A as predicted by the genetic profile (Fig 3.).

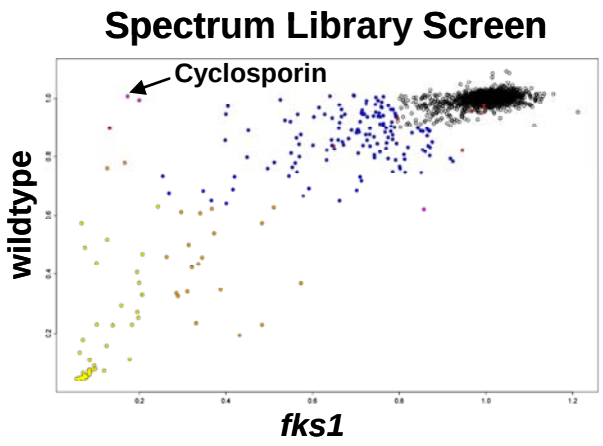

Figure 3. A pilot screen identifies Cyclosporin A chemical synthetic lethality in an *fks1Δ* mutant strain. Parental (y axis) or *fks1Δ* (x axis) strains were screened against the 2000 compound Spectrum library in duplicate at a concentration of 20μM in HEPES-buffered SC media. Residual growth is calculated as the ratio % absorbance compared to an internal DMSO control for each strain. Mean values for each screen are plotted (circles). Cyclosporin A is denoted by an arrow.

Since Cyclosporin A appeared as one of the strongest hits in an *fks1Δ* background, I went on to determine if Cyclosporin A would produce a unique profile corresponding to its genetic interactions. I screened several additional known *cnb1*-interacting mutants among a panel of mutants that had no known genetic interactions. The screen was able to reliably identify the known genetic interactors while none of the other strains exhibited any significant bioactivity in the presence of Cyclosporin A (Fig. 4).

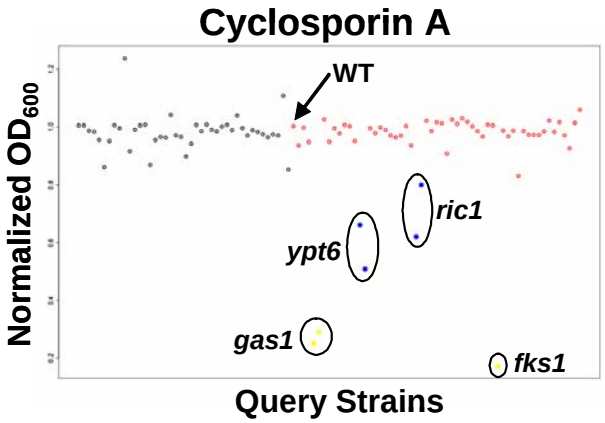

Figure 4. . Sensitized strains (circles) were screened in duplicate against the Spectrum small molecule library (red) at a final concentration of 20μM. Absorbance (OD_{600} y-axis) was determined 18 hours post-inoculation. Mean values are plotted. Inactive compounds are show with open circles. Bioactive compounds are

shown in blue (Z score >4). Cytotoxic compounds are shown in yellow (<2SD from cytotoxic control). *CNB1* genetic interactors are denoted with ovals. Note: *gas1, ypt6 and ric1* mutants were slow growing and were measured at two time points to capture the maximum dynamic range (both time points are shown). Wildtype reference strain is denoted with an arrow.

Conclusions

I was able to successfully map the genetic interactions surrounding the yeast kinase *SCH9* and, to a lesser degree, the phosphatase *Tep1*. There are a number of challenges that still exist in defining the genetic network surrounding any given gene in the *Saccharomyces cerevisiae* genome. Neither method employed, SGA or DSLAM, provided comprehensive (or overlapping) coverage. The inability to pick out previously determined interactions indicates false negative interactions remain an Achilles heel of both assays. In spite of these shortcomings, genome-wide genetic interaction mapping is a powerful tool for elucidating genetic networks.

The proof of principle assays, using both the analogue sensitive kinase allele of *SCH9* (year one) and the *CNB1*-Cyclosporin A model system; demonstrate the validity of the surrogate synthetic lethal approach. It is clear that sensitized strains can be used as reporters for small molecule inhibitors of non-essential proteins. The sensitized strains allow interrogation of chemical space that would be invisible in wild type cells. One of the features of the system is the ability to enhance the phenotype from a subtle cellular effect that may be difficult to quantify in a high throughput format, such as cell size, to a death phenotype that has a wide dynamic range and is easy to measure.

While the small molecule screens were ultimately unsuccessful in finding a specific inhibitor of Sch9, they were a valuable resource and ultimately bore fruit for a related project. The data collected from the differential screen of the 50,000 compound Maybridge small molecule library allowed us to filter and select a subset of compounds that have bioactivity against yeast. We built a sub library of 1500 compounds which we are naming the SacchPAC, for Saccharomyces Pharmacologically Active Compounds that will be made available to the yeast community for pilot phenotype screens.

Unfortunately, since the preliminary small molecule screens produced no viable leads I was unable to proceed with aims 3 and 4 as outlined in the statement of work. The problems with the genetic assays that were encountered originally were ultimately resolved; however, the chemical space of the library that was available did not encompass backbones that specifically interfered with Sch9 function. The results with the Cyclosporine A proof-of-principle make me hopeful that the *TEP1* genetic interactor screen will be successful when the network is fully defined.

Training Accomplishments

Travel Awards:

Cold Spring Harbor Laboratory Scholarship ($1500), Cold Spring Harbor Laboratory Yeast Genetics Course, Cold Spring Harbor, New York, July 22-Aug. 11, 2003

Keystone Symposia travel scholarship ($1000), New Advances in Drug Discovery, Keystone, Colorado, March 21-26, 2004

Mount Sinai Hospital Research Training Center Travel Award (CDN$500), AACR Cancer Targets and Chemotherapeutics, Geneva, Switzerland, Sept. 28-Oct. 1, 2004

Courses

Cold Spring Harbor Laboratory Yeast Genetics Course, Cold Spring Harbor, New York, July 22-Aug. 11, 2003

Collaborations

Eric Brown, Ph.D., Director, McMaster University HTS Facility, McMaster University, Hamilton, Ontario, Canada. Provided training on Biomek FX instrument. Provided venue for initial high throughput screen.

Leigh Revers, D. Phil., Operations Manager, Mount Sinai Hospital High Throughput Facility, Mount Sinai Hospital, Toronto, Ontario, Canada. Collaborated on programming and validation for fully automated high throughput screening method.

Jan Wildenhain, B.Sc., Database engineer/programmer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada. Collaborated on generation of chemi-informatics database platform.

Chao-Jun Li, Ph.D., Professor, Department of Chemistry, McGill University, Montreal, Canada. Provided compounds for testing chemical genetic fingerprinting using barcode microarrays.

Key Research Accomplishments:

- Developed automated high-throughput screening platform for cell-based yeast assay.
- Performed SGA/DSLAM to determine synthetic lethal genetic interactions with *sch9Δ*.
- Performed SGA/DSLAM to determine synthetic lethal genetic interactions with *tep1Δ*.
- Confirmed genetic interactions with *SCH9* and *TEP1*
- Demonstrated SLC proof-of-principle using *sch9-AS*.
- Conducted large-scale high-throughput SLC screen of parental DS and sensitized *rpl9bΔ*strains.
- Developed chemi-informatic database.

Reportable Outcomes

Review: Sharom JR, **Bellows DS**, Tyers M. (2004). “From large networks to small molecules.” Curr Opin Chem Biol. **8**(1):81-90

Review: **Bellows DS,** Tyers M**.** (2004) “Chemical genetics hits reality.” Science. **306**(5693):67-8

Poster: EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics”, Geneva, Switzerland, 28 September-1 October, 2004. “The Synthetic Lethal Trap: a novel genetic assay to probe for specific chemical inhibitors of non-essential proteins using HTS” David S. Bellows, Paul Jorgensen and Mike Tyers. The Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto (see appendix for abstract)

Platform Presentation: “Era of Hope” Department of Defense Breast Cancer Research Meeting, Philadelphia, Pennsylvania, June 8-11, 2005. Concurrent Symposium 8 “The Synthetic Lethal Trap: a novel genetic assay to probe for specific chemical inhibitors of non-essential proteins using HTS” David S. Bellows, The Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto (see appendix)

Database: “ChemGrid” a chemi-informatic resource. http://medbase.mshri.on.ca/~jw/chem_grid/

Small Molecule Library: SachhPAC bioactive set derived from Maybridge Library screen (a screening resource of 1500 compounds with bioactivity against *S. cerevisae*).

Faculty Position: Lecturer (Assistant Professor) in Cellular and Molecular Biology, Victoria University of Wellington, Wellington, New Zealand commencing October, 2006.

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Pan, X., D. S. Yuan, et al. (2004). "A robust toolkit for functional profiling of the yeast genome." Mol Cell **16**(3): 487-96.

Park, E.-C. and H. R. Horvitz (1986). "MUTATIONS WITH DOMINANT EFFECTS ON THE BEHAVIOR AND MORPHOLOGY OF THE NEMATODE CAENORHABDITIS ELEGANS." Genetics **113**(4): 821-852.

Parsons, A. B., R. L. Brost, et al. (2004). "Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways." Nat Biotech **22**(1): 62-69.

Winzeler, E. A., D. D. Shoemaker, et al. (1999). "Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis." Science **285**(5429): 901-6.

Appendix

Abstract: Era of Hope, Department of Defense Breast Cancer Research Meeting
Philadelphia, Pennsylvania June 8-11, 2005

THE SYNTHETIC LETHAL TRAP: A GENERAL APPROACH FOR SCREENING SMALL-MOLECULE PROTEIN INHIBITORS USING GENETIC TRIANGULATION IN THE YEAST SACCHAROMYCES CEREVISIAE

David S. Bellows, Paul Jorgensen, and Mike Tyers
Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada
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Our purpose was to develop a novel chemical-genetic screening strategy to identify small-molecule inhibitors of any non-essential protein using a surrogate synthetic lethal (SL) phenotype. Synthetic lethality is a form of genetic enhancement in which two mutations are lethal in combination, but the corresponding individual mutants are viable. We hypothesize that SL genetic interactions can be used as reporters for drug-based inhibition of any non-essential protein function. If protein inactivation using a small molecule is functionally equivalent to a loss-of-function mutation, then a specific protein inhibitor must satisfy all the genetic SL interactions associated with the corresponding gene. For example, if inactivation of genes A & B are SL, then a cell missing gene A will die in the presence of a small-molecule inhibitor of protein B. Genome-wide genetic screens have been developed to simultaneously determine all the SL interactions for a given gene deletion in yeast. Preliminary screens reveal ~30 SL interactions/gene. Furthermore, each gene appears to have a unique pattern of interactions, or genetic fingerprint. Thus, a collection of sensitized strains, each containing individual deletions that are SL with a target gene, should act as a trap for specific small-molecule inhibitors of the target protein. If sensitized-strain SL interactions overlap only with the target gene, positive hits must be genetically linked to the target gene.

Using the Synthetic Genetic Array (SGA) approach, we determined the SL partner genes for a deletion of SCH9, a kinase involved in size control in yeast and the closest homologue to the human oncogene, Akt. As a proof-of-principle, we constructed an analog-sensitive (AS) SCH9 allele that is specifically inhibited by the ATP analog NM-PP1. AS-SCH9 strains carrying gene deletions that are SL with sch9Δ were inviable in the presence of NM-PP1 while the single mutants, or the AS strain alone, grew in the presence of the drug, demonstrating the validity of the approach. We then developed a high-throughput liquid comparative growth assay and screened the 50,000-molecule Maybridge library for Sch9 inhibitors using our chemical-genetic trap. Potential hits are being confirmed and subjected to subsequent rounds of screening against non-overlapping sensitized strains. Confirmed hits will be analyzed in secondary phenotype and biochemical screens.

The surrogate genetic phenotype of this novel approach allows in vivo identification of inhibitors of previously intractable targets such as membrane proteins or genes whose deletion gives no observable phenotype. This method will accelerate the translation of small molecules into clinical therapies by providing a means to rapidly and efficiently assay for inhibitors of virually any non-essential target.

The U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0471 supported this work.

Abstract: EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics", Geneva, Switzerland, 28 September-1 October, 2004

The synthetic lethal trap: A general approach for screening small-molecule protein inhibitors using genetic triangulation in the yeast *Saccharomyces cerevisiae*.

David S. Bellows, Paul Jorgensen and Mike Tyers, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada

We set out to develop a novel chemical-genetic screening strategy to identify small-molecule inhibitors of any non-essential protein using a surrogate synthetic lethal (SL) phenotype. Synthetic lethality is a form of genetic enhancement in which two mutations are lethal in combination, but the corresponding individual mutants are viable. If protein inactivation using a small molecule is functionally equivalent to a loss-of-function mutation, then a specific protein inhibitor should satisfy all the genetic SL interactions associated with the corresponding gene. We hypothesize that SL genetic interactions can be used as reporters for drug-based inhibition of any non-essential protein function. For example, if inactivation of genes A & B are SL, then a cell missing gene A should die in the presence of a small-molecule inhibitor of protein B. Genome-wide genetic screens have been developed to simultaneously determine all the SL interactions for a given gene deletion in yeast. Preliminary screens reveal ~30 SL interactions/gene. Furthermore, each gene appears to have a unique pattern of interactions, or genetic fingerprint. Thus, a collection of sensitized strains, each containing individual deletions that are SL with a target gene, should act as a trap for specific small molecule inhibitors of the target protein. If sensitized strain SL interactions overlap only with the target gene, positive hits must be genetically linked to the target gene.

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(DOD Breast Cancer Research Program DAMD17-03-1-0471)